

Research on Ion Transport Across Microbial Membranes

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FOREWORD

This is the one hundred and sixtieth of a series of reports designed to present accounts of progress in saline water conversion with the expectation that the exchange of such data will contribute to the long-range development of economical processes applicable to large-scale, low-cost demineralization of sea or other saline water.

Except for minor editing, the data herein are as contained in the reports submitted by TRW Space Technology Laboratories, under Contract No. 14-01-0001-416, covering research carried out through May 24, 1965. The data and conclusions given in this report are essentially those of the Contractor and are not necessarily endorsed by the Department of the Interior.

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SUMMARY

This study is designed to investigate the mechanisms involved in the transport of inorganic ions across the cytoplasmic membranes of Serratia marcescens and S. marnorubra. The two bacteria are closely related genetically but S. marnorubra is considerably more tolerant to NaCl and requires salt for optimum growth. A systematic and comparative investigation of certain salt transport characteristics was begun to determine the relationship between the divergent salt tolerance properties of the two bacteria and membrane structure and function.

The permeability and transport rates of the chloride salts of sodium, lithium, potassium, magnesium, strontium and calcium were examined by suspending resting cells in hypertonic salt solutions. The resulting plasmolysis was measured by an increase in light scattered by the cell suspension. The immediate increase in light scattering was followed by a gradual decline in light scattering, attributable to salt accompanied by water re-entering the cell (deplasmolysis). Cells of S. marcescens were more permeable to monovalent cations than to bivalent cations; potassium was transported more rapidly than any other cation. The phase of growth at which the cells were harvested influenced both the magnitude of the plasmolytic response and the rate at which cations permeated the cells. The youngest cells produced the largest response and were the most resistant to deplasmolysis. The transport of salts is an active process and appears to be dependent upon the energy derived from the aissimilation of the endogenous reserve material, glycogen. The glycolytic inhibitors, iodoacetate and mercuric ion, completely retarded or inhibited the rate of salt transport. The respiratory inhibitors, 2,4 dinitrophenol, arsenite and cyanide, accelerated the rate of salt uptake. The rate of transport was suppressed when the cells respired formate. These results suggest that the inhibition of respiration and oxidation of formate resulted in a Pasteur effect which produced accelerated transport mediated by glycolytic processes. Hydrogen ion concentration modified the response to the salts - the higher the pH, the greater the plasmolytic response, and the faster the rate of transport. Salts were transported more slowly when oxygen was absent than under aerobic conditions, possibly reflecting the influence of the redox potential of the suspending medium. Shaking the cell suspension vigorously in air resulted in enhanced rates of salt transport, however, these results can not yet be explained.

Preliminary studies showed that cells of S. marnorubra were markedly more impermeable to monovalent cations than S. marcescens when grown in a medium lacking NaCl and examined under essentially identical conditions. Further studies will be necessary to correlate the nature of the observed differences in membrane permeability and the internal osmotic pressure of the respective organisms.

INTRODUCTION

With the recent emphasis by the Office of Saline Water on utilizing membrane processes such as electrodialysis and reverse osmosis to desalinate water economically, the study of biological mechanisms of ion transport has become increasingly important. It is anticipated that information derived from biological studies will lead to the development of more efficient membranes capable of operating for prolonged periods at low temperatures, low pressures and with minimal energy requirements.

Few organisms are capable of existing over a wider range of ionic environments than the bacteria. Bacteria are capable of growth at salt concentrations ranging from practically no salt to saturated brine. In addition, the ionic composition of their cytoplasm is generally different from that of their environment. For example, all bacteria accumulate K^+ and exclude Na^+ against a concentration gradient. Because of these characteristics and their relatively simple structure, bacteria make excellent models for the study of ion transport phenomena.

Bacteria regulate their intracellular ion composition by the existence of a permeability barrier located at the cell surface. The permeability barrier is composed of a lipoprotein membrane and possibly a second component as well. Since the permeability barrier not only selectively regulates the internal composition of the cell but because of its location must tolerate a greater range of ionic conditions than the intracellular cytoplasm itself, it is logical that studies of the nature and function of the cytoplasmic membrane should lead to a greater understanding of the mechanisms involved in ion transport.

Our approach to this problem consists of a comparative study of the ion transport characteristics of two bacteria, Serratia marcescens and Serratia marnorubra, genetically similar in almost every respect except in their tolerance to salt concentration. S. marnorubra is considerably more tolerant to NaCl than S. marcescens. It appears reasonable, therefore, to suggest that the divergent salt tolerance properties of these two bacteria can be attributed primarily to some function of their cytoplasmic membranes rather than to some strictly metabolic or physiological function. It is recognized, however, that metabolic and other physiological activities must contribute to ion transport as well. It is believed that a systematic and comparative investigation of the respective ion transport characteristics of these two bacteria when cultured and suspended in similar and contrasting salt environments, should furnish further insight into the mechanism of ion transport.

Our experimental approach rests upon the observation by numerous workers that an increase in the external osmotic concentration of the medium surrounding the

cell results in a corresponding increase in the amount of light scattered by the cell suspension. It has been shown that the magnitude of this change depends not only upon the osmotically active substance but also upon the species of bacteria, its history of growth and environmental conditions. It is generally agreed that light scattering changes result from plasmolysis, i.e., loss of intracellular water due to the ionic concentration gradient. Exposure of cells to osmotically active substances such as inorganic salts produces this effect. More light is scattered because the resulting reduced internal osmotic pressure causes the cytoplasm to compress and thereby increases the refractive index of the cell. Also contributing in part to the increase in light scattering is the irregular surface of the cell wall created by the compressed cytoplasm distorting the cell wall. Increases or decreases in the amount of light scattering by cells suspended in hypertonic solutions can thus be used to rapidly and sensitively measure the rate of substances entering and exiting through the cell membrane.

Using this technique, studies were directed toward characterizing inorganic ion transport phenomena with the two experimental bacteria. Neither bacteria had been examined for this aspect of their physiology previous to this investigation. The chloride salts of Na^+ , K^+ , Li^+ , Mg^{++} , Ca^{++} and Sr^{++} were selected for study. By using a common anion, the influence of the cations could more readily be ascertained. Because of the close similarity between S. marcescens and S. marinorubra, the majority of experiments during the first year of this investigation were performed with S. marcescens, the more widely studied of the two. When sufficient data is collected to characterize the nature of ion transport with this organism, it is intended to then compare the behavior of S. marinorubra under identical and controlled conditions. Some preliminary data showing the contrast in salt transport characteristics will appear in this report.

MATERIALS AND METHODS

Bacteria

The bacteria used for all experiments were Serratia marcescens HY and Serratia marinorubra S10. Both organisms were kindly furnished by Dr. W. Belser, University of California, Riverside.

Medium

Both bacteria were grown in a medium containing:

glycerol	20 g
yeast extract	1 g
peptone	2 g
distilled water	1000 ml
final pH	6.8

NaCl was added to the medium for some experiments.

Growth conditions

Cultures were grown with shaking at 30°C using a reciprocal shaker. Growth was determined by measuring turbidity at 660 m μ with a Beckman DB spectrophotometer.

Preparation of resting cells

The cultures were harvested after an appropriate growth period by centrifugation at 4°C, washed 2 times with distilled water and resuspended in 0.05 M tris (hydroxymethyl) aminomethane (Tris), pH 6.8. Before final suspension in buffer, the cells were filtered through Whatman No. 1 filter paper to remove large particles and debris. The suspension was adjusted to an optical density of 0.28 - 0.30 at 660 m μ prior to addition of inorganic salts and organic substrates. The cell suspensions were maintained under aerobic conditions throughout the experimental procedures.

Light scattering measurements

A volume of 3 ml of cell suspension was added to a Brice-Phoenix 10x10 mm (ID) light scattering cuvette. Reagents were delivered with a micro-syringe.

Light scattering was measured with a Model 2000-D Brice-Phoenix Universal Light Scattering Photometer (Phoenix Precision Instrument Co., Phila., Pa.), using the 546 m μ emission maximum of a mercury arc and a photocell at 90° to the incident light. Measurements were recorded on a strip-chart recorder (ServoRiter, Model 304A, Texas Instruments Inc., Houston, Texas). The term "% light scattered" used in some graphs refers to the amount of light scattered by plasmolyzed cells over the amount of light scattered by normal cells. The maximum amount of light scattered after the addition of inorganic salts is arbitrarily designated as 100% unless otherwise indicated.

Respiration measurements

Oxygen consumption by the cell suspensions was measured with a Clark oxygen microelectrode (Beckman Instruments, Inc., Palo Alto, Calif.) placed directly

into the cuvette containing the suspension. The rate of oxygen utilization was recorded on a strip-chart recorder (Varian GC-10, Varian Associates Inc., Palo Alto, Calif.).

Biochemical reaction tests

Biochemical reactions were determined with Key Rapid Fermentation Tablets (Key Scientific Products Co., Los Angeles, Calif.).

RESULTS AND DISCUSSION

Biochemical reactions of *S. marcescens* and *S. marinorubra*

The nomenclature and taxonomy of the genus *Serratia* has been studied by many workers but the proper classification for members of this genus remains controversial. A recent study of Colwell and Mandel (1965) using Adansonian analysis (computer techniques) and DNA base composition determinations found that *S. marinorubra* could not be distinguished from *S. marcescens*. It was concluded that *S. marcescens* is the only species in the genus. According to their classification scheme, *S. marinorubra* is actually a salt-tolerant strain of *S. marcescens*. For the purposes of our study, however, the classification of these two organisms is not critical, because the important feature of the two bacteria selected for this study is that they be similar in their biochemical and physiological characteristics but dissimilar in their tolerance to salt. To compare biochemical characteristics, the organisms were tested for their ability to ferment a random variety of carbohydrates. Several other standard tests were included as well. The biochemical reactions produced by *S. marcescens* and *S. marinorubra* are presented in Table 1.

The identical responses to all the biochemical reactions tested tends to corroborate the conclusions reached by Colwell and Mandel regarding the classification of these two bacteria. For the purpose of identification, however, separate nomenclature will be used in this report.

Tolerance to NaCl

In Figure 1, the contrasting effect of salt concentration on growth of the two bacteria over a 24-hour period can be seen clearly. Growth of *S. marcescens* in nutrient broth containing NaCl decreased linearly over a range of 0-6% NaCl; no growth occurred above 4% NaCl. Growth of *S. marinorubra* was fairly equivalent over a range of 0-4% NaCl; a limited amount of growth occurred even in 10% NaCl.

TABLE I

Biochemical Reactions of S. marcescens and S. marinorubra

<u>Substrate or test</u>	<u>S. marcescens</u>	<u>S. marinorubra</u>
Glucose	+	+
Lactose	+	+
Maltose	+	+
Sucrose	+	+
Mannitol	+	+
Salicin	+	+
Adonitol	+	+
Arabinose	+	+
Cellobiose	+	+
Dextrin	+	+
Dulcitol	+	+
Galactose	+	+
Inositol	+	+
Inulin	+	+
Fructose	+	+
Melibiose	+	+
Raffinose	+	+
Rhamnose	+	+
Sorbitol	+	+
Trehalose	+	+
Xylose	+	+
Urease	-	-
Gelatin hydrolysis	+	+
Oxidase	+	+

+ production of acid(from carbohydrates)

- no reaction

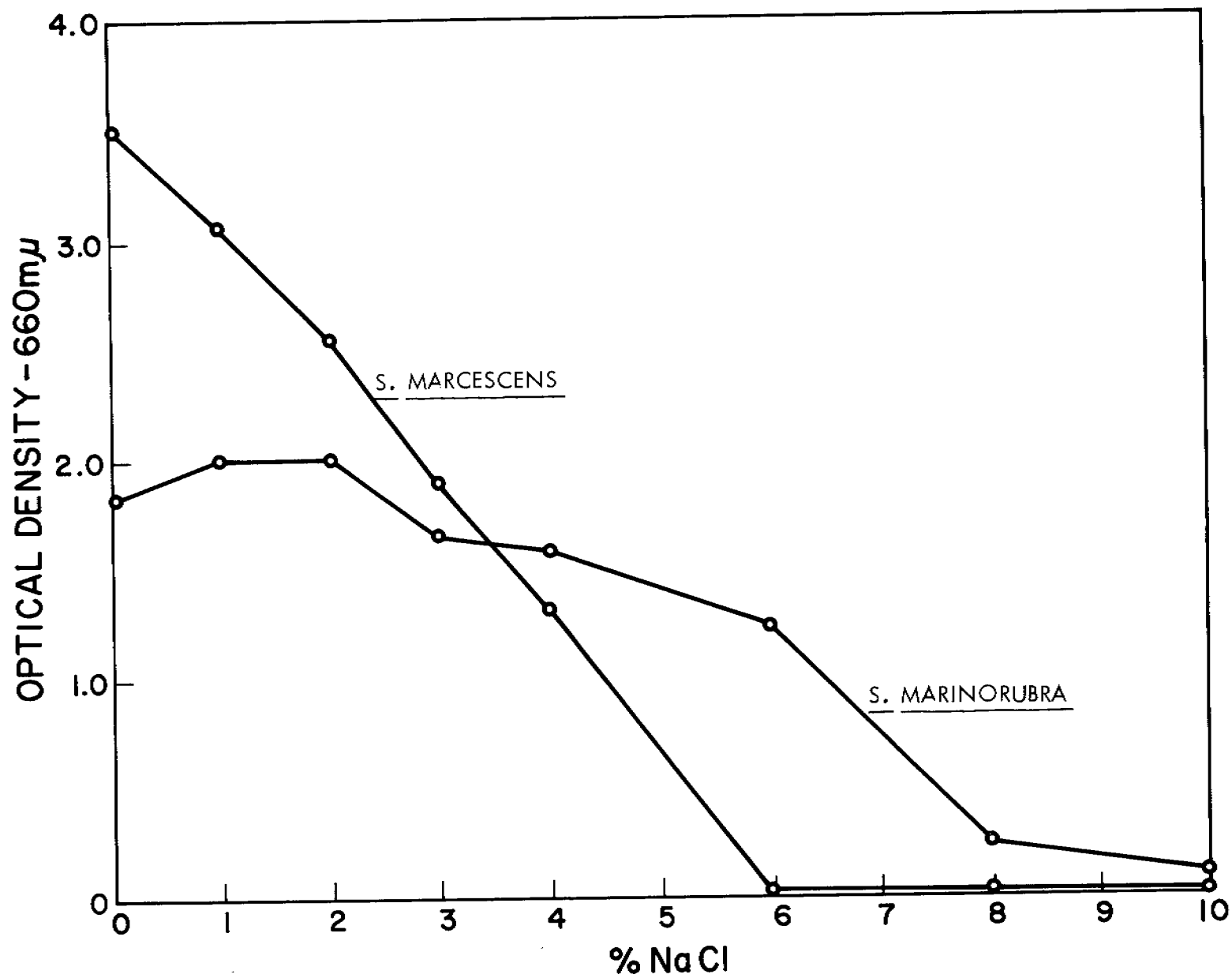


FIG. 1: Growth in Nutrient Medium Containing NaCl

S. marcescens grew optimally in nutrient broth lacking NaCl while with S. marinorubra, optimum growth occurred at 1-2% NaCl. Growth of the two bacteria was essentially equal between 3 to 4% NaCl, but S. marcescens grew considerably better than S. marinorubra at the lower concentrations of NaCl.

Growth rates in nutrient broth

The rate of growth of S. marcescens and S. marinorubra in nutrient broth (lacking NaCl) was determined. The comparative growth rates are illustrated in Figure 2. The rate of growth of S. marinorubra lagged behind S. marcescens for the first 6 hours following inoculation, but was approximately the same during the exponential phase of growth. The lag exhibited by S. marinorubra suggests that an adaption period is required before a maximum growth rate can be reached. Total growth of S. marcescens was significantly greater than S. marinorubra in this medium, which indicates the requirement for salt before S. marinorubra can grow optimally.

Growth rates in 3% NaCl

The rate of growth between the two bacteria in nutrient broth containing 3% NaCl was compared. In Figure 3, it can be seen that S. marinorubra grew at a higher rate and more extensively during an 8-hour period than S. marcescens in this medium. Growth rates were similar during the first 4 hours of growth, but were widely divergent from 4 to 6 hours following inoculation. Since total growth was equivalent after 24 hours growth (see Fig. 1), it appears from the slope of the S. marinorubra growth curve that the rate of growth sharply decreased after 8 hours.

Titration of cell suspensions with inorganic salts

A cell suspension of S. marcescens was titrated with NaCl and $MgCl_2$ over the range of 0.08 to 0.32 M and the magnitude of light scattered was measured. The salt solutions at a concentration of 5 M were added progressively at 45-second intervals. Figure 4 shows that 0.08 M of the respective salts increased light scattering of the suspension 75% while increasing salt concentrations produced proportionally smaller increases. A similar type of response was produced by the chloride salts of K^+ , Li^+ , Ca^{++} and Sr^{++} . The plasmolysis plateau of the titration curve, i.e., the salt concentration at which plasmolysis is maximal, was 0.24 M with NaCl and 0.16 M with $MgCl_2$. Because an excessive concentration of salts can cause the cells to hyperplasmolyze, a condition from which the cells are incapable of recovering, it is preferable to select a salt concentration which does not produce the maximum plasmolytic response. Since a salt concentration of 0.08 M produced the largest single increase in light scattering and was still considerably less than the plateau, this concentration was selected for routine experiments unless indicated otherwise.

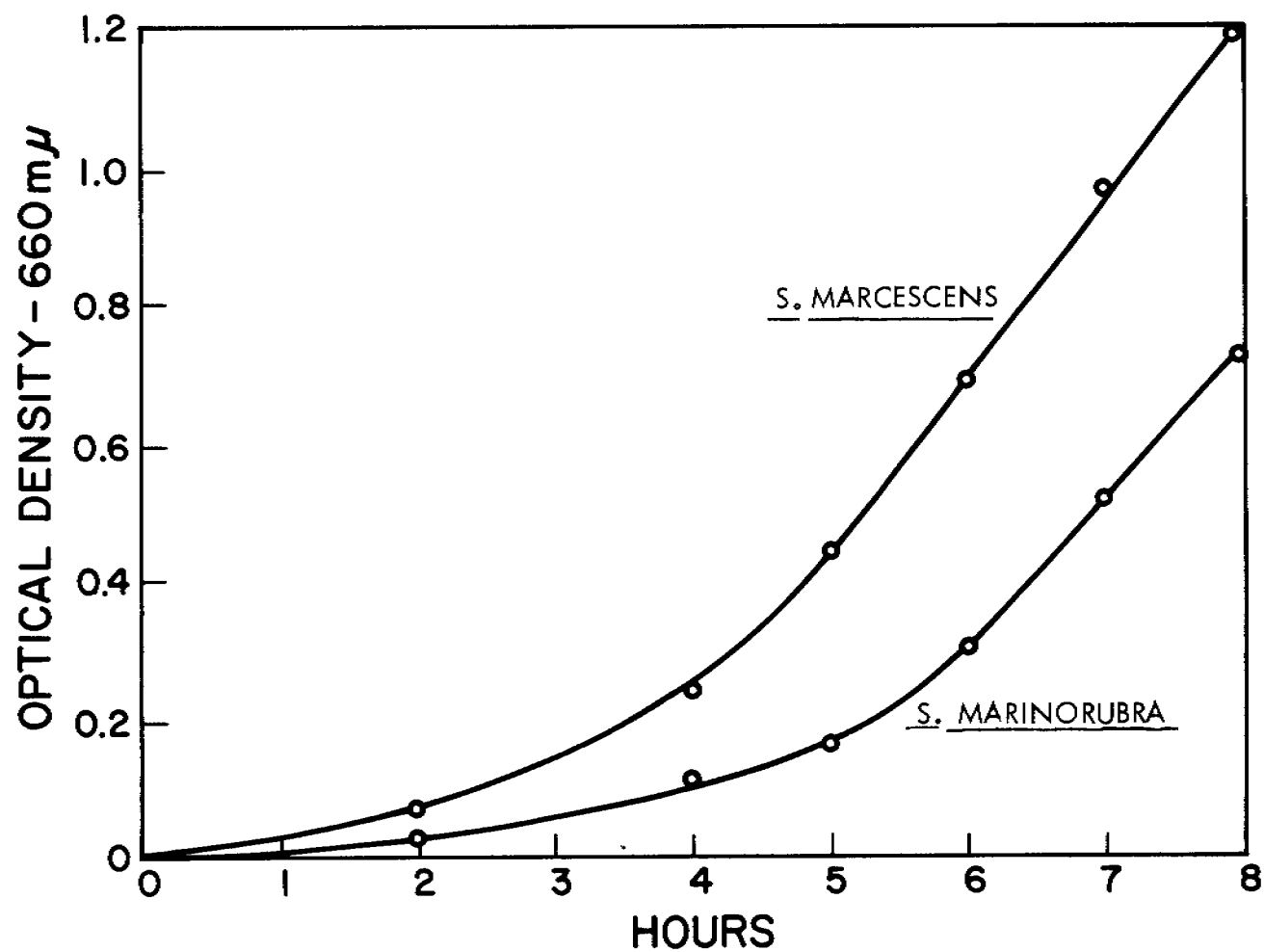


FIG. 2: Growth of *S. marcescens* and *S. marnorubra* in Nutrient Medium

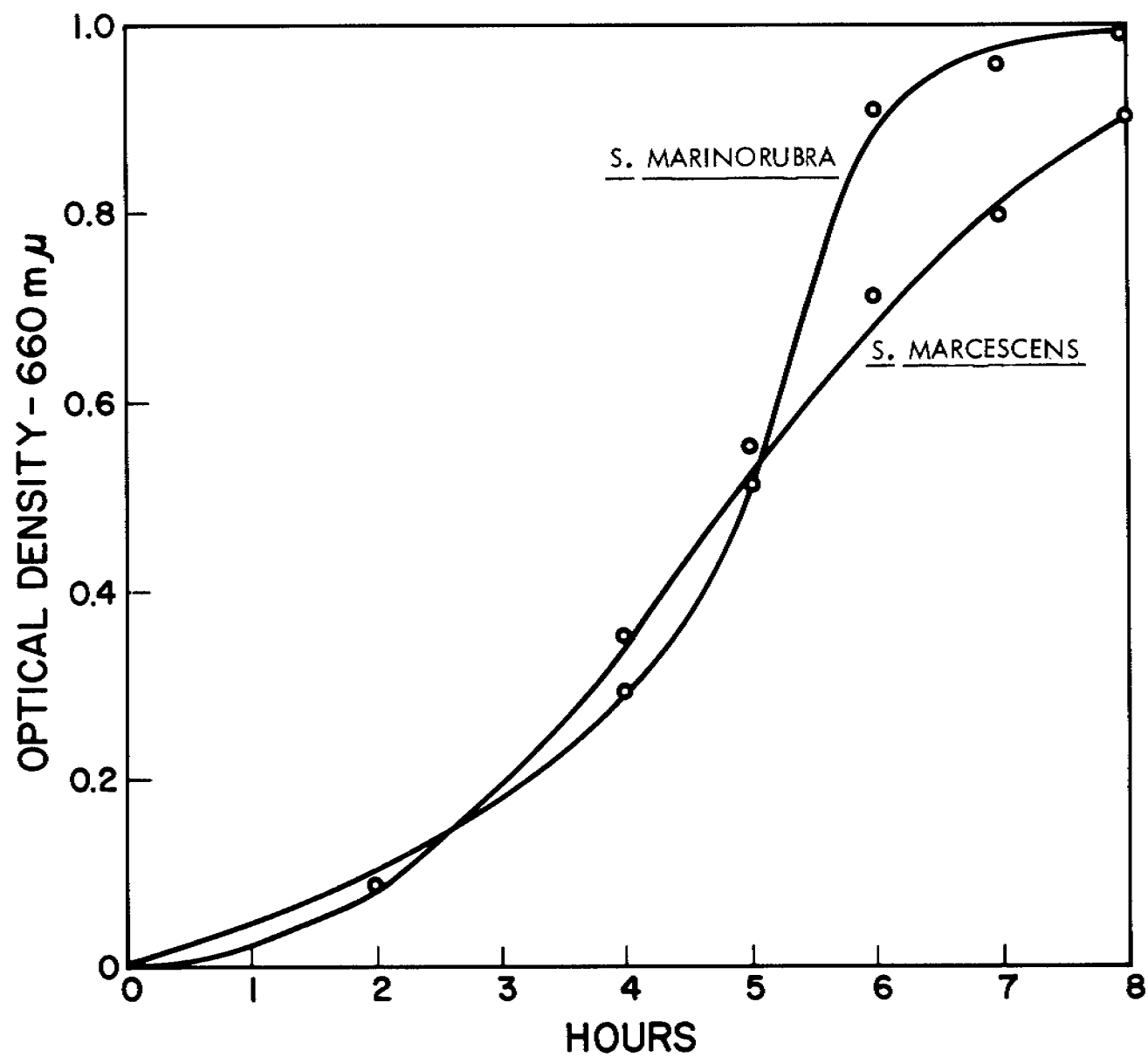


FIG.3: Growth in Nutrient Medium Containing 3% NaCl

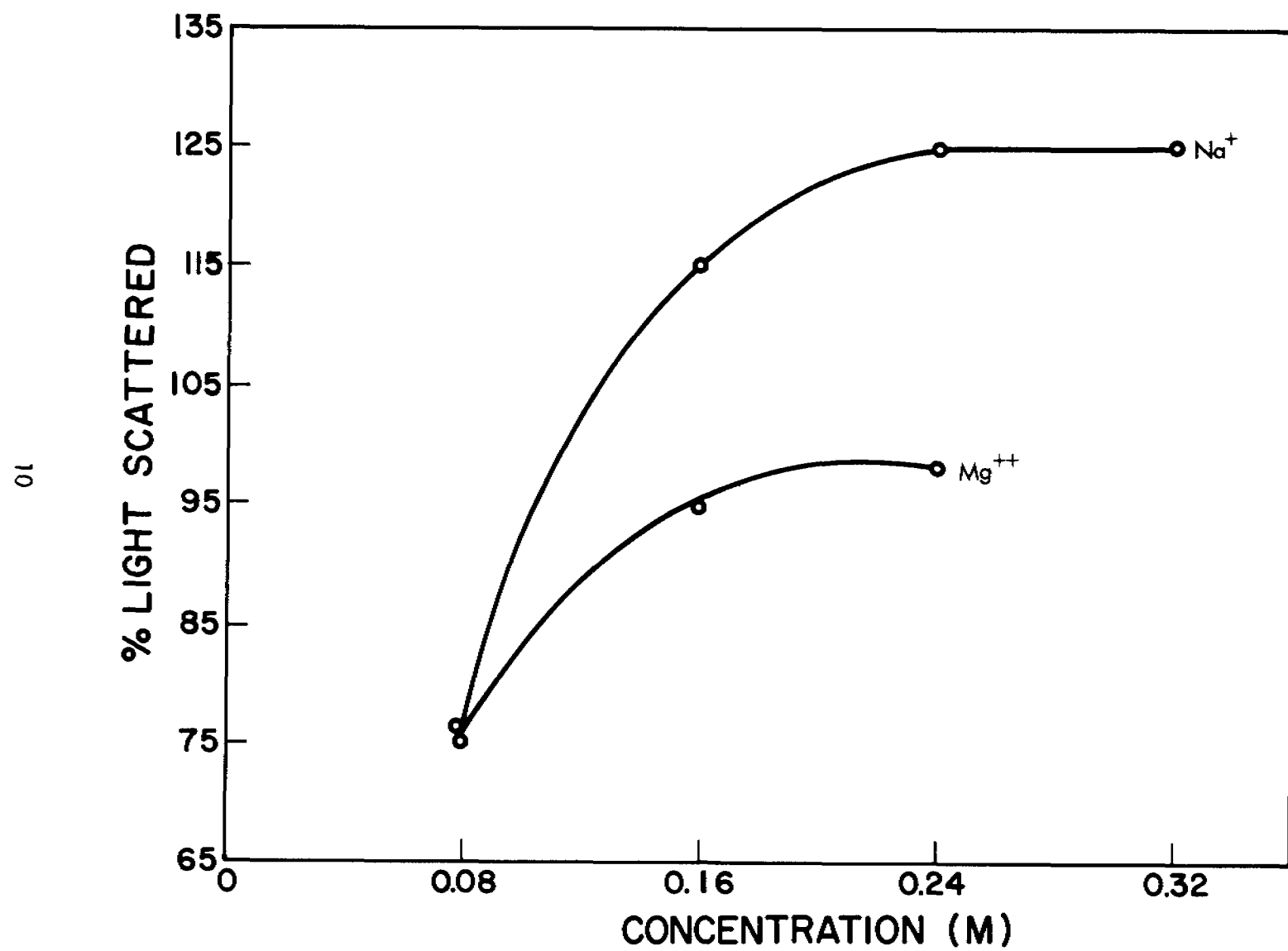


FIG. 4: Titration of Cell Suspension with NaCl and MgCl₂

Rates of deplasmolysis

Figure 5 shows that the immediate increase in light scattering (plasmolysis) after the addition of the chloride salts of Na^+ , K^+ and Li^+ was followed by a gradual decrease in light scattering (deplasmolysis) with time. Deplasmolysis occurred the most rapidly with KCl ; with LiCl and NaCl the rate was approximately the same. In the presence of the chloride salts of Mg^{++} , Ca^{++} and Sr^{++} , no deplasmolysis was evident over the 7.5 minute period immediately following the addition of the salts. However, after a period of 15-30 minutes, the cells began to slowly deplasmolyze until after several hours the cells approached or returned completely to normal. In the case of the monovalent cations, complete deplasmolysis occurred in less than one hour. Thus the initial plasmolytic condition is relatively transient in the case of the six cations examined, although the cells are considerably more resistant to deplasmolysis with bivalent cations than with monovalent cations.

Since the deplasmolysis rates were examined in quiescent solutions, it was of interest to determine whether reduction of concentration gradients by agitation would alter the rate of deplasmolysis, i.e., was the process diffusion controlled. A rapidly vibrating needle was immersed in a cuvette containing NaCl plasmolyzed cells and the rate of deplasmolysis compared. It was found that agitation did not increase the rate of deplasmolysis over that of quiescent cell suspensions. This suggests that the rate limiting step in ion transport is biological and occurs at the surface of the cell -- probably at the cytoplasmic membrane itself.

The decrease in light scattering can be attributed to salt re-entering the cell accompanied by water. The transport of water is considered to be passive and follows the concentration gradient produced by diffusion of the salt. With electrolytes, it is difficult to determine whether it is the cation or anion that transports through the cell membrane because ions must move in pairs to maintain electroneutrality. If a cation diffuses through a membrane, it must be balanced by an equal number of anions in the same direction or an equivalent number of cations of the same charge in the opposite direction. In the case of chloride salts, most studies have indicated that the anion follows the cation through the membrane to satisfy the electrochemical gradient. It is the cation, then, that is influenced by some type of driving force, such as active transport.

Of the two sequential events which follow the addition of salts to the cell suspension, i.e., the initial increase in light scattering attributable to plasmolysis and the slow decline in light scattering attributable to salts accompanied by water re-entering the cell, it is deplasmolysis that is of greater interest from the standpoint of these ion transport studies. The immediate plasmolytic effect

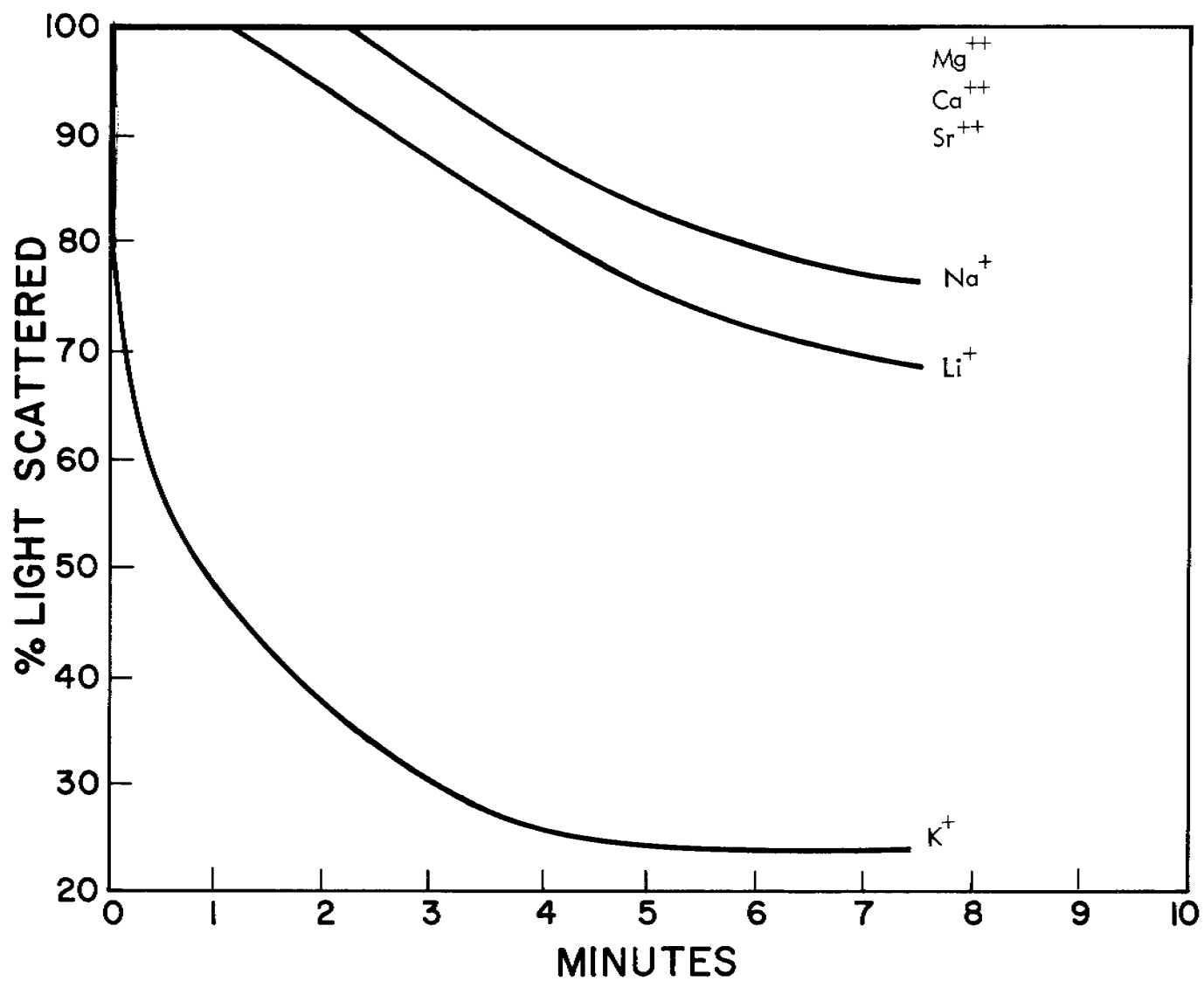


FIG. 5: Light Scattering Changes Following Addition of Inorganic Salts

produced by salts appears to be primarily an osmotic response to hypertonic solutions, while deplasmolysis may involve metabolic energy. A key question in the field of ion transport concerns the relationship of cellular metabolism to transport and the mechanism by which the energy derived from metabolism couples to the transport of substances (active transport). The literature regarding active transport has been comprehensively reviewed (Rothstein, 1959a) and will not be reported here except as it is directly relevant to the results obtained in these studies. Numerous theories have been offered to explain transport phenomena, but due to the complexity of biological systems, the problem is far from resolved.

There is little doubt that bacteria employ different mechanisms to transport monovalent and bivalent cations and that cells discriminate between cations by a variety of transporting systems (Rothstein, 1959a). The monovalent cations have been studied more extensively than bivalent cations. The general picture has been complicated by the fact that competition exists between cations and that certain cations stimulate the transport of other cations. Probably more studies have been made with K^+ than any other single cation. It is well known that K^+ is accumulated by all cells and that many enzymes have a specific requirement for K^+ . It is not surprising then, that many studies have shown that K^+ stimulates and is essential for the uptake of numerous organic and inorganic compounds (Krebs et al., 1957; Mitchell and Moyle, 1959; Packer and Perry, 1961). The extremely rapid rate at which K^+ is transported by *S. marcescens* cells is most likely a reflection of the special role of this cation. Studies of Na^+ transport are also numerous, but less is known about the mechanisms involved in its transport than K^+ . Because of this and the relatively high concentration of Na^+ in sea water, the majority of our studies were carried out with NaCl.

With respect to the bivalent cations, Mg^{++} was studied for very much the same reasons. Investigations with this cation have been hampered in the past because no convenient radioisotope of Mg^{++} is available.

Influence of phase on growth on salt transport

Wide variations in preliminary experiments led to a study in which the influence of the growth phase on light scattering was examined. It was found that the rate or "age" at which the cells were growing when harvested determined both the magnitude of the plasmolytic response and the rate of deplasmolysis. Figure 6 shows a typical 24-hour sigmoid growth curve for *S. marcescens* grown in nutrient broth. Figure 7 illustrates the magnitude of the plasmolytic response to the chloride salts of Na^+ , K^+ , Li^+ , Ca^{++} , Sr^{++} and Mg^{++} when cells were harvested from three different stages in the exponential phase of growth. The greatest increase in light scattering occurred with cells harvested just subsequent to the lag phase

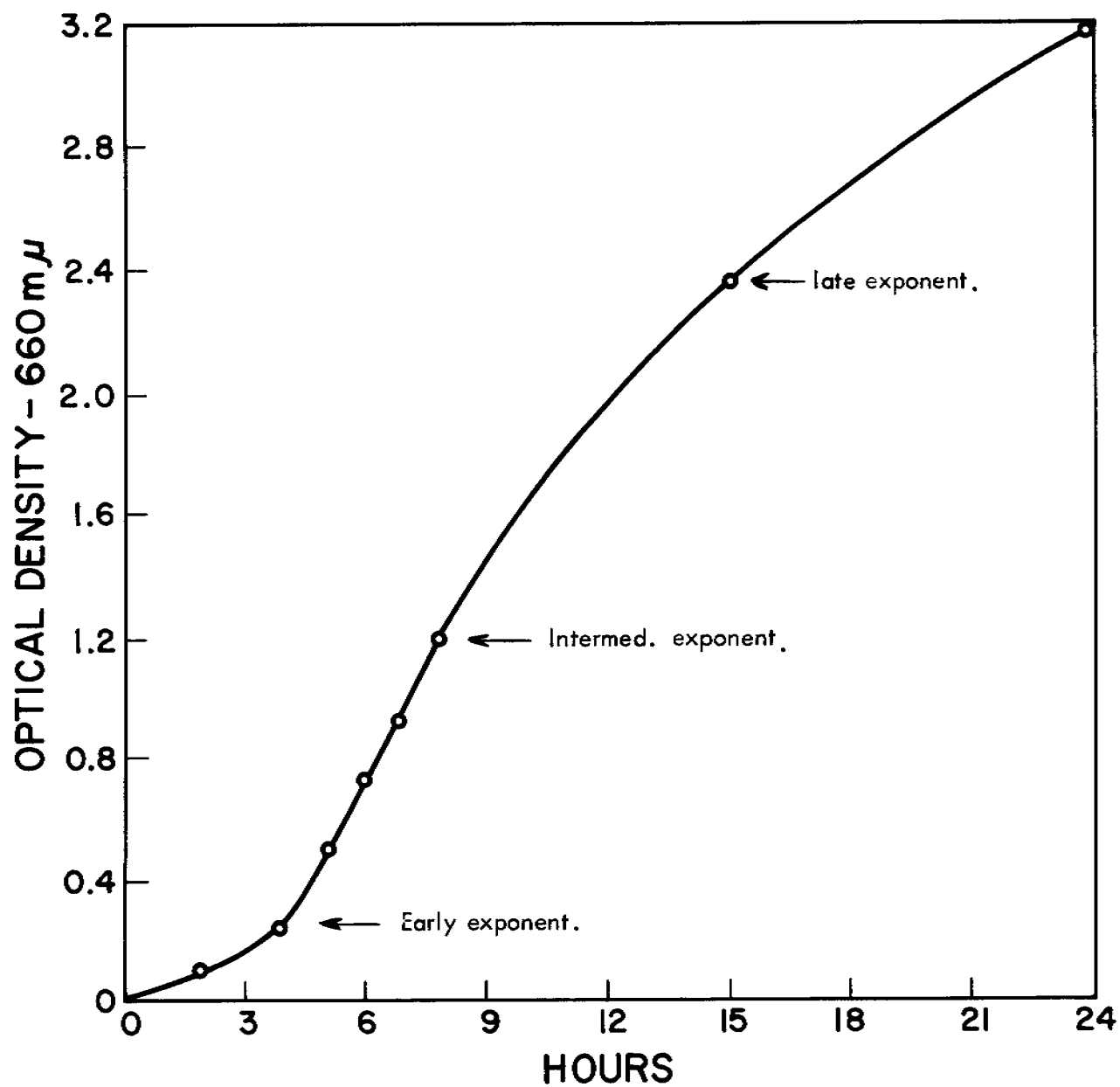


FIG. 6: Growth of *S. marcescens* in Nutrient Medium

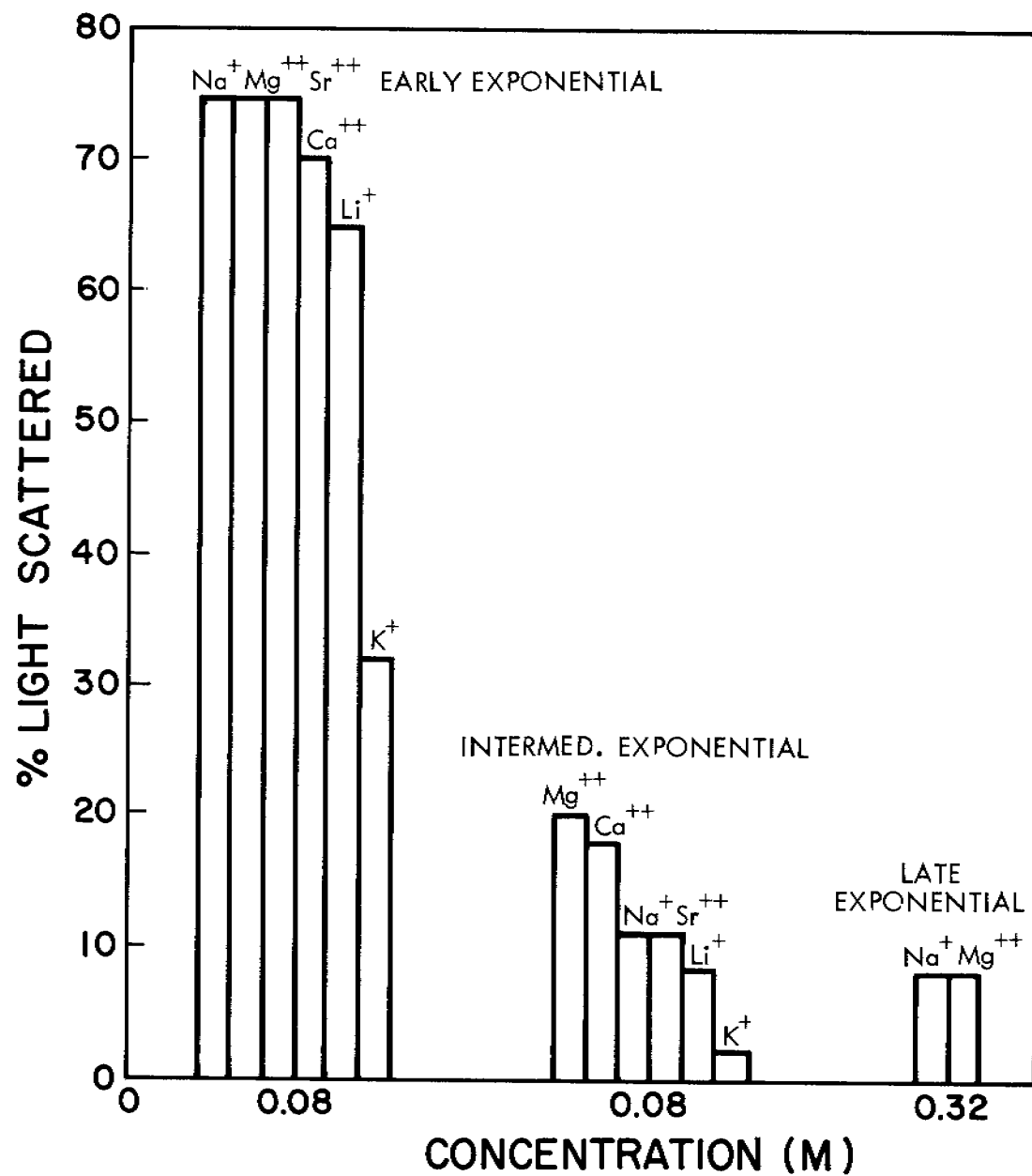


FIG. 7: Influence of Age on Magnitude of Light Scattering

(early exponential phase). With cells harvested in the late exponential phase, four times the concentration of $MgCl_2$ did not produce as great a response as with intermediate exponential phase cells.

Figure 8 shows the comparative rates of deplasmolysis between cells harvested in the early exponential phase and intermediate exponential phase. The younger cells were resistant to deplasmolysis with $MgCl_2$ during a 7.5 minute period while a rapid decrease in light scattering was evident with the older cells. This relationship was also true of the chloride salts of Na^+ , K^+ , Li^+ , Sr^{++} and Ca^{++} .

The influence of the phase of growth on osmotic behavior has also been observed by other workers with different bacteria. Mager, et al. (1956) found that plasmolysis of Pasteurella tularensis was maximal with cells harvested at the end of the lag phase, decreased during the exponential phase and reached a constant level in the stationary phase. Winlow and Walker (1939) reported that the more rapid the rate of growth of Escherichia coli at the time of harvesting, the more osmotically fragile the cells.

There are several possibilities to explain these differences in permeability other than the most obvious explanation that the permeability of the cell membrane changes with the phase of growth. The internal cytoplasmic osmotic pressure, the rate of metabolism, the adhesion of the cytoplasmic membrane to the cell wall and tensile strength of the cell wall may be determining factors and are likely to vary with the "age" of the cell.

Because the younger cells produced the greatest plasmolytic response, cells were harvested from the early exponential phase for routine experiments. The optical density ranged from 0.20 - 0.24 at 660 $m\mu$. Variations in the turbidity of the culture will be so indicated.

Metabolic inhibitor studies

These experiments were designed to investigate whether the entry of salts into the cell is an active energetic process involving metabolism or whether salt transport is mediated by passive diffusion; or a combination of both. Studies with bacteria and other cells have shown that the transport of such cations as Na^+ and K^+ is an active process and involves metabolic energy in the form of adenosine triphosphate (ATP). Transport against a concentration gradient using metabolic energy allows the cells to accumulate substances from an external environment where the concentration of the substance is low, to excrete toxic or unwanted compounds and most likely to maintain a normal cell volume. Although the most recent concepts of active transport can be found in the literature, a brief description of one of the more popular schemes is useful for this report. It is believed that the penetrating

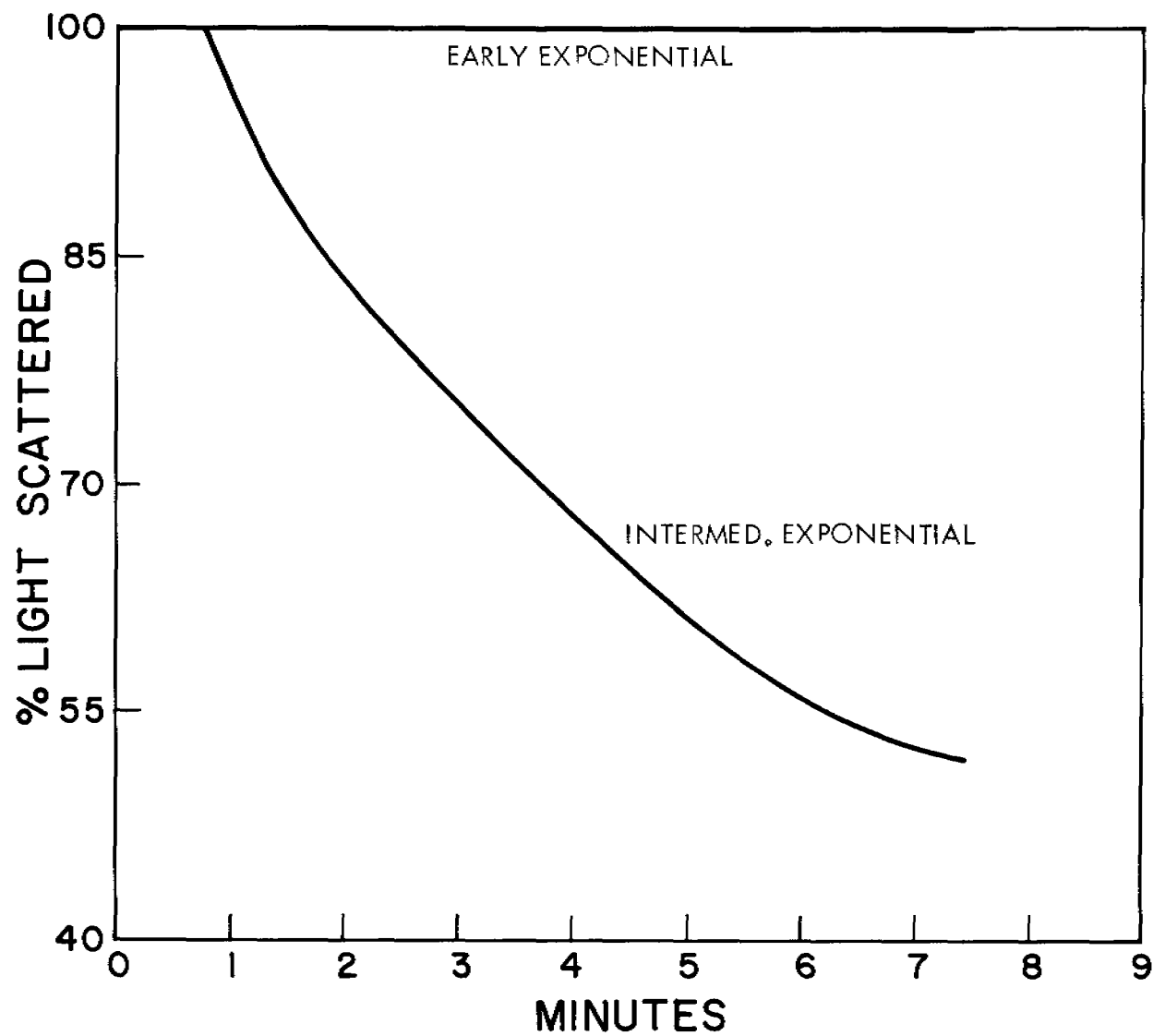


FIG. 8: Influence of Growth Phase on Mg^{++} Transport

ion or molecule combines with an enzyme-like carrier, sometimes called a permease or translocase, located in the membrane. The carrier is modified on one side of the membrane by a process which involves the energy derived from ATP to a form which has a high affinity for the penetrating substance. The newly created carrier-complex shuttles across the membrane where it is subjected to another reaction involving ATP which releases the molecule. The carrier then transports back across the membrane, either alone or in combination with some other type of molecule and the cycle is then repeated.

A classical approach to determining whether ion transport is dependent upon metabolism and understanding the enzymatic pathways involved is the use of metabolic and enzyme inhibitors. It can be deduced by the judicious application of certain inhibitors, for example, whether glycolysis and/or respiration is contributing to ion transport.

In the first series of experiments, the influence of two glycolytic inhibitors, iodoacetate (sodium salt) and mercuric chloride, on the rate of deplasmolysis was examined. Mercuric chloride, at a concentration of 4.2×10^{-4} M completely prevented deplasmolysis in the presence of NaCl, LiCl and even KCl. The effective concentration of the inhibitor was so small that even the residual mercuric chloride remaining in the cuvette after normal rinsing was sufficient to exert complete inhibition of deplasmolysis with a fresh aliquot of cells.

Sodium iodoacetate (2×10^{-2} M) produced a similar inhibition of deplasmolysis with the chloride salts of Na^+ , K^+ and Li^+ , but did not produce as complete an effect as mercuric. Figure 9 shows that iodoacetate reduced the rate of deplasmolysis over that of the control by 42% after a preincubation period of 30 minutes.

Both mercuric and iodoacetate inactivate enzymes by combining with -SH groups. Many key enzymes contain sulfhydryl groups including alcohol dehydrogenase, succinic dehydrogenase and phosphoglyceraldehyde dehydrogenase, all important to carbohydrate metabolism (glycolysis). The sulfhydryl groups on the last named enzyme, a key enzyme in the glycolytic pathway, is particularly susceptible to these types of inhibitors. These results agree with those of Rieder (1951), who reported that iodoacetate inhibited glucose uptake in yeast. Avi-Dor et al. (1956), on the other hand, found that both inhibitors increased the rate of salt uptake with Pasteurella tularensis. Their results may indicate a fundamental difference in transport mechanisms between the two bacteria although any analogy is complicated because conditions were not precisely the same.

The results obtained indicate that salt uptake involves a metabolic process and that this process is dependent upon glycolysis. Since the cells were not provided with an exogenous energy source, they must have obtained metabolic energy from

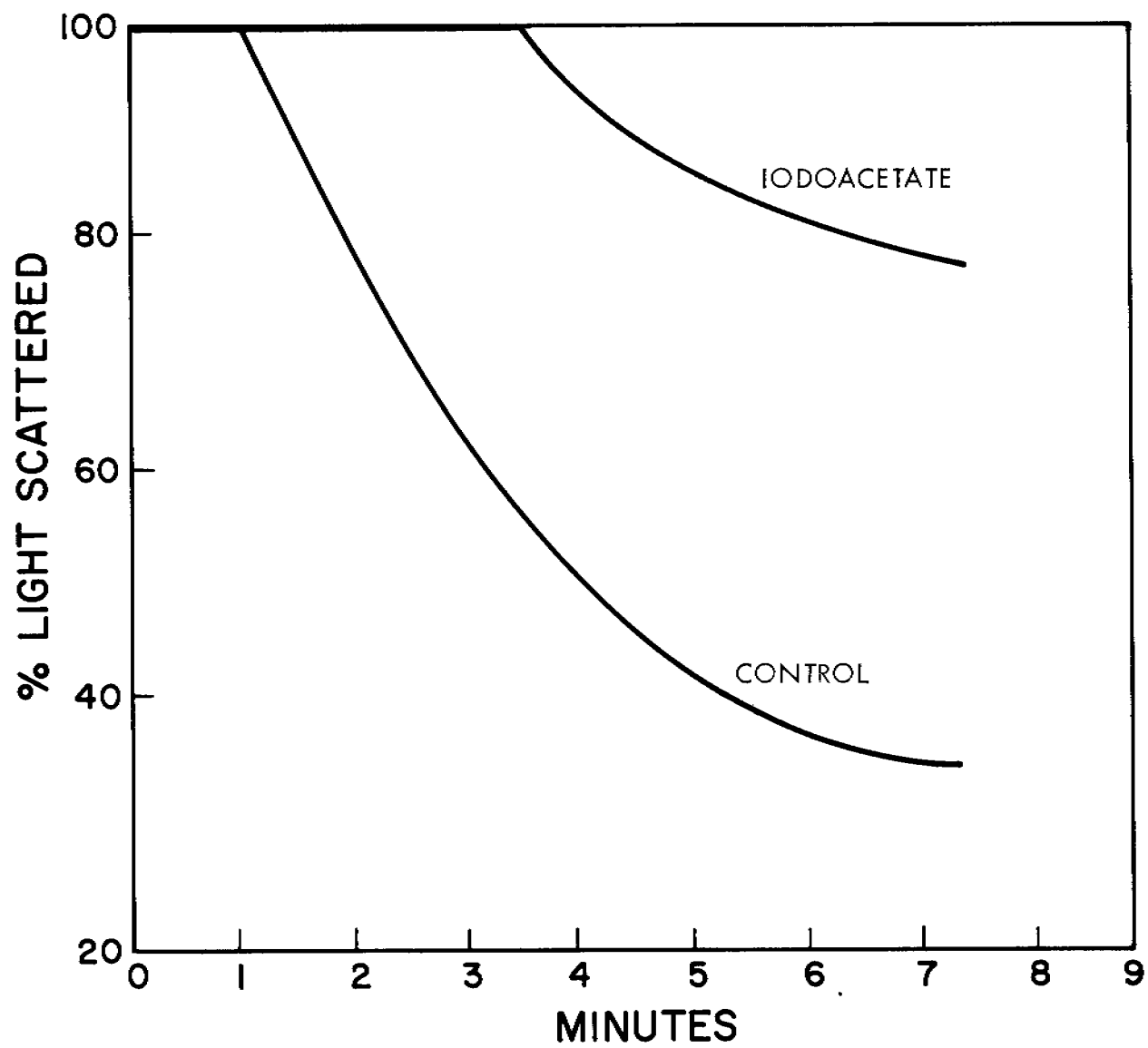


FIG. 9: Effect of Iodoacetate on Na⁺ Transport

endogenous reserves, (the oxygen electrode was used to demonstrate that resting cell suspensions slowly consumed oxygen). *S. marcescens* stores the polysaccharide, glycogen, as a primary reserve material and, in the absence of exogenous carbon and energy sources, dissimilates glycogen via the glycolytic pathways. Since oxygen is available to the resting cells throughout the experimental period, metabolism can proceed further through the tricarboxylic acid cycle (TCA) and electron transport system (ETS). The TCA cycle and the ETS constitute the primary respiratory processes of the cell. However, since respiration depends upon glycolysis when endogenous glycogen is the substrate, resting cells are unable to respire when the glycolytic pathway is blocked by inhibitors. The oxygen electrode was used to confirm this experimentally.

The influence of respiratory inhibitors on salt transport was examined in the next series of experiments. These consisted of the sodium salt of cyanide, an inhibitor of cytochrome oxidase, a key enzyme in the electron transport system; the sodium salt of arsenite, an inhibitor of TCA cycle enzymes and the sodium salt of 2,4 dinitrophenol (DNP), an uncoupler of oxidative phosphorylation. DNP is not strictly a respiratory inhibitor because it permits respiration, and at some concentrations even increases the rate of respiration of oxidizable substrates but prevents the generation of ATP in the process. Consequently, the cell cannot obtain energy from respiration.

Figure 10 shows that all three inhibitors had a similar effect on salt transport and that unlike the glycolytic inhibitors, they accelerated the rate of deplasmolysis. Even cells plasmolyzed with Mg^{++} and normally resistant to deplasmolysis, rapidly underwent a decrease in light scattering when incubated with cyanide. Cyanide and arsenite were shown to completely inhibit respiration while DNP slightly stimulated the rate of respiration.

Bernheim (1963), working with *Pseudomonas aeruginosa*, reported that cyanide prevented the transport of phosphate into cells. This organism does not, however, store glycogen as a reserve material, but stores poly-beta-hydroxybutyric acid, a fatty acid polymer. Rieder (1951) observed that cyanide increased the rate of glucose uptake in yeast. DNP has been shown to generally inhibit the transport of organic substrates such as amino acids and hexoses with bacteria (Bovell et al., 1963; Gale, 1951). Thus, the influence of the respiratory inhibitors reported in the literature has been variable and appears to be dependent upon the conditions of the experiment and the choice of organism.

The results suggest that the respiratory inhibitors exerted a Pasteur effect on metabolism. The Pasteur effect states that the presence of oxygen suppresses glycolysis. The most widely accepted explanation of the Pasteur effect is that respiration is so efficient that the adenylic acid and ADP available in aerobic

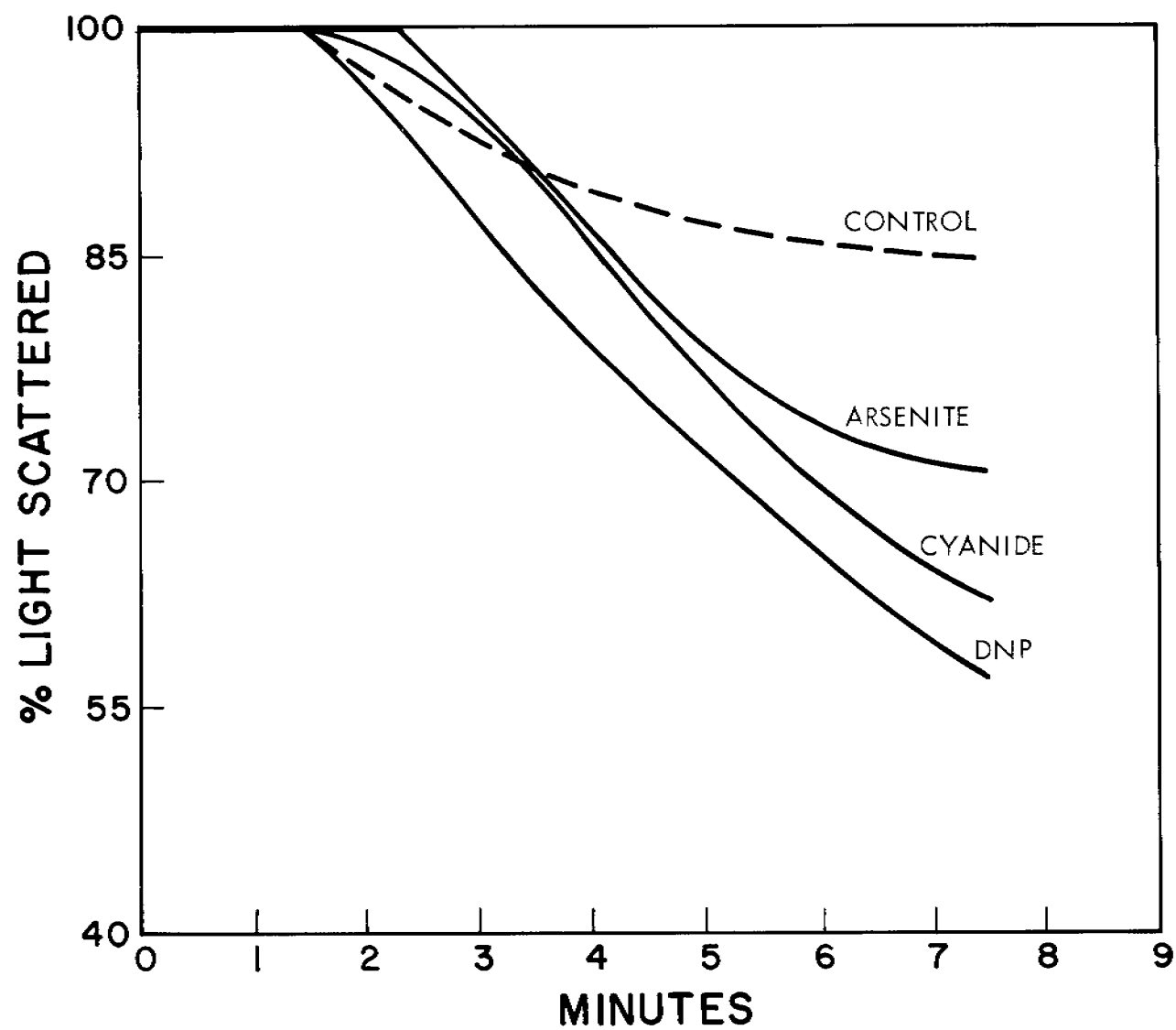


FIG. 10: Effect of Respiratory Inhibitors on Na^+ Transport

cells is rapidly converted to ATP, and therefore none of the adenylic acid and ADP is available for the phosphorylation of triose phosphate, a key glycolytic intermediate. Thus, carbohydrate metabolism is inhibited because of a deficit of ADP. Conversely, suppression of respiration will increase the rate of glycolysis. To illustrate this concept, the relationship between metabolic inhibition and ATP generation and the relative effects of the respective inhibitors on the rate of salt transport obtained in this study are summarized in Table 2.

TABLE 2
Effect of Inhibitors on Metabolism and Salt Transport

	Production of ATP by		Rate of	
	Glycolysis	Respiration	Respiration	Salt Transport
Normal cells	ATP	ATP	+	+
DNP (3×10^{-5} M)	ATP ⁽⁺⁾	-	++	+++
NaCN (1×10^{-2} M)	ATP ⁽⁺⁾	-	-	+++
NaAsO ₃ (1×10^{-2} M)	ATP ⁽⁺⁾	-	-	++
ICH ₂ CO ₂ Na (2×10^{-2} M)	-	-	-	-*
HgCl ₂ (trace)	-	-	-	-

* 42% decrease in rate

The indications are, that if the Pasteur effect is operative in these cells, the rate of salt transport may be accelerated as a result of increased glycolytic activity. There is a growing body of evidence to show that glycolysis is involved in some manner with ion transport. Scott et al. (1951) showed that the energy derived from the anaerobic dissimilation of glycogen in yeast can be used to accumulate potassium against a concentration gradient. Packer and Perry (1961) found that cations are transported into cells of *E. coli* with the energy obtained from the anaerobic metabolism of hexoses. While it is true that the relationship of glycolysis to cellular structure is still not clear, cytological studies have indicated that the glycolytic enzymes appear to be located in the periphery of bacterial and yeast cells (Rothstein, et al., 1959). The zone in which the glycolytic system is located appears to be directly beneath the cytoplasmic membrane, so that coupling to active transport could transpire, although the precise nature of the coupling is not known. Nor is it known exactly how glycolysis contributes to the transport of ions.

It should be mentioned that interpretations derived from inhibitor studies with respect to ion transport phenomena must be approached with caution because inhibitors, particularly heavy metals, sometimes combine with enzyme and protein groups located at the cell membrane itself and alter the permeability of the membrane. For example, yeast cells exposed to certain concentrations of mercuric have been shown to become completely leaky and lose intracellular potassium, amino acids and other compounds (Rothstein, 1959b). Therefore, it is not always possible to differentiate between the primary influence of the inhibitor on cellular metabolism and the secondary effect of the inhibitor on the permeability of the cell membrane. An evaluation of our results indicates, however, that the inhibitors used did not seriously alter the permeability of the membrane since the magnitude of the plasmolytic response to salts was similar to normal cells. If the inhibitors caused a breakdown in the membrane as a permeability barrier, plasmolysis would have been considerably impaired.

Another inherent problem relating to inhibitor studies is that some inhibitors, again heavy metals in particular, tend to be non-specific when used with living cells and inhibit many enzyme systems. Specificity is usually concentration-dependent. For example, iodoacetate is generally considered to be a specific inhibitor for glyceraldehyde dehydrogenase and can, therefore, specifically block the glycolytic pathway, but the literature reveals that at least 55 enzymes are known to be inhibited to some degree by iodoacetate. DNP, on the other hand, is one of the more specific inhibitors known. This does not mean that the use of inhibitors is useless in living cells, but conclusions drawn from such studies must be treated with reserve and correlated with data obtained from other types of approaches.

Influence of organic substrates on salt transport

As a result of some of the problems inherent in the use of inhibitors, we were prompted to seek additional approaches to determining the major metabolic pathways involved in salt transport. Of particular interest was another method of demonstrating the suggested Pasteur effect.

It has been shown by Bovell et al. (1963) that the addition of respirable organic compounds to plasmolyzed cell suspensions produces two types of effects. If the cells are capable of actively accumulating the compound (accompanied by water), the osmotic concentration of the cells increases and thereby the cells scatter less light (deplasmolyze). If, however, the cells are freely permeable to the compound and the compound equilibrates in equal concentrations within and without the cell, no change in light scattering or osmotic pressure of the cells occurs. It has also been shown that the energy derived from the respiration of externally provided organic compounds can be used to transport inorganic salts into the cell (Bovell and Packer, 1963). It appeared reasonable to assume, then, that if a Pasteur effect

is in fact produced by respiratory inhibitors, respiration of an externally provided organic compound should decrease the rate of glycolysis since, as discussed earlier, an increase in cellular respiration will suppress glycolysis. A reduction in the rate of glycolysis should, in turn, reduce the rate of deplasmolysis.

It was necessary to provide the cells with an energy source that was not actively accumulated, since the process of accumulation, in itself, decreases light scattering. Therefore, a variety of hexoses, amino acids, organic acids and miscellaneous organic compounds were examined to find a respirable substrate that passively equilibrated across the cell membrane. The technique consisted of adding NaCl to a final concentration of 0.08 M to the cells and then adding the organic substrate (final concentration 30 mM), at a point when the rate of deplasmolysis was minimal, generally 7-10 minutes after the addition of NaCl.

The results of these studies are presented in Table 3.

TABLE 3

Influence of Organic Respirable Substrates on Salt Transport

<u>Substrate *</u>	<u>Effect on Rate of Transport</u>
Glucose	+
Mannose	+
Fructose	+
Alanine	+
Sodium glutamate(L)	+
Glycine	+
Sodium pyruvate	-
Sodium succinate	-
Sodium lactate	+
Glycerol	+
Ammonium formate	-

-
- + accelerated rate of Na^+ transport
 - no effect on rate of Na^+ transport
 * final concentration was 30 mM

It can be seen that only formate, pyruvate and succinate did not decrease light scattering. All other compounds apparently were actively accumulated. Bovell and Packer (1963) found that resting cells of E. coli were freely permeable to formate and that both inorganic and organic compounds were transported by the energy provided by the oxidation of formate. Formate was selected, then, to determine whether its respiration would produce a Pasteur effect. Since formate does not decrease light scattering by itself, NaCl and formate were added

simultaneously to the cell suspension. Figure 11 shows that, as predicted, the rate of deplasmolysis in the presence of formate was significantly decreased. Respiration of formate was shown to begin without a lag, which is characteristic of freely permeable substrates.

Although these results provide additional evidence of the involvement of the glycolytic pathway in transporting inorganic salts into the cell, considerably more work is necessary to elucidate the function and relationship of glycolysis to salt transport. Still to be determined is the reason why glycolysis accelerates salt transport in view of the small amount of ATP generated by fermentation reactions compared to respiration. It would be interesting to compare salt transport rates in cells completely depleted of glycogen to cells containing normal and larger than normal reserves of glycogen. Techniques are available to regulate the amount of endogenous reserves. Several attempts were made during this study to exhaust reserve glycogen by the common method of prolonged aeration, however, it was found that after several hours the cells began to lyse and underwent changes in permeability.

Another approach, not yet attempted, is to grow cells in the presence of small concentrations of fluoride. Fluoride prevents yeast and probably bacteria from converting glucose to glycogen (Nickerson and Chung, 1952). Alternately, cells can be grown in a medium in which the carbon source is limited, thus minimizing the storage of reserve materials.

Effect of pH on salt transport

The dependence of ion transport on pH appears to vary according to the test organism and experimental conditions. Avi-Dor et al. (1956) reported that the uptake of NaCl by Pasteurella tularensis was independent of pH values between 6 and 8. Henneman and Umbriet (1964) found that both the magnitude of turbidity and rate of deplasmolysis increased with resting cells of E. coli suspended in NaCl solutions. With yeast cells, Conway and Moore (1954) observed that "as the pH of the medium is raised Na⁺ uptake increased because of reduced competition by H⁺, which has a high affinity for the transport systems."

To examine the influence of pH with S. marcescens cells, the transport of Na⁺ was examined at pH 6.5, 7.3, 8.0 and 8.5. As shown in Figure 12, the results agree with those obtained by other workers with E. coli and yeast, i.e., the lower the pH, the slower the rate of transport. The rate of deplasmolysis was over three-fold more rapid at pH 8.5 than at 6.5. Moreover, the amount of light scattered increased with increasing pH, with the exception of pH 8.5. These results are shown in Table 4.

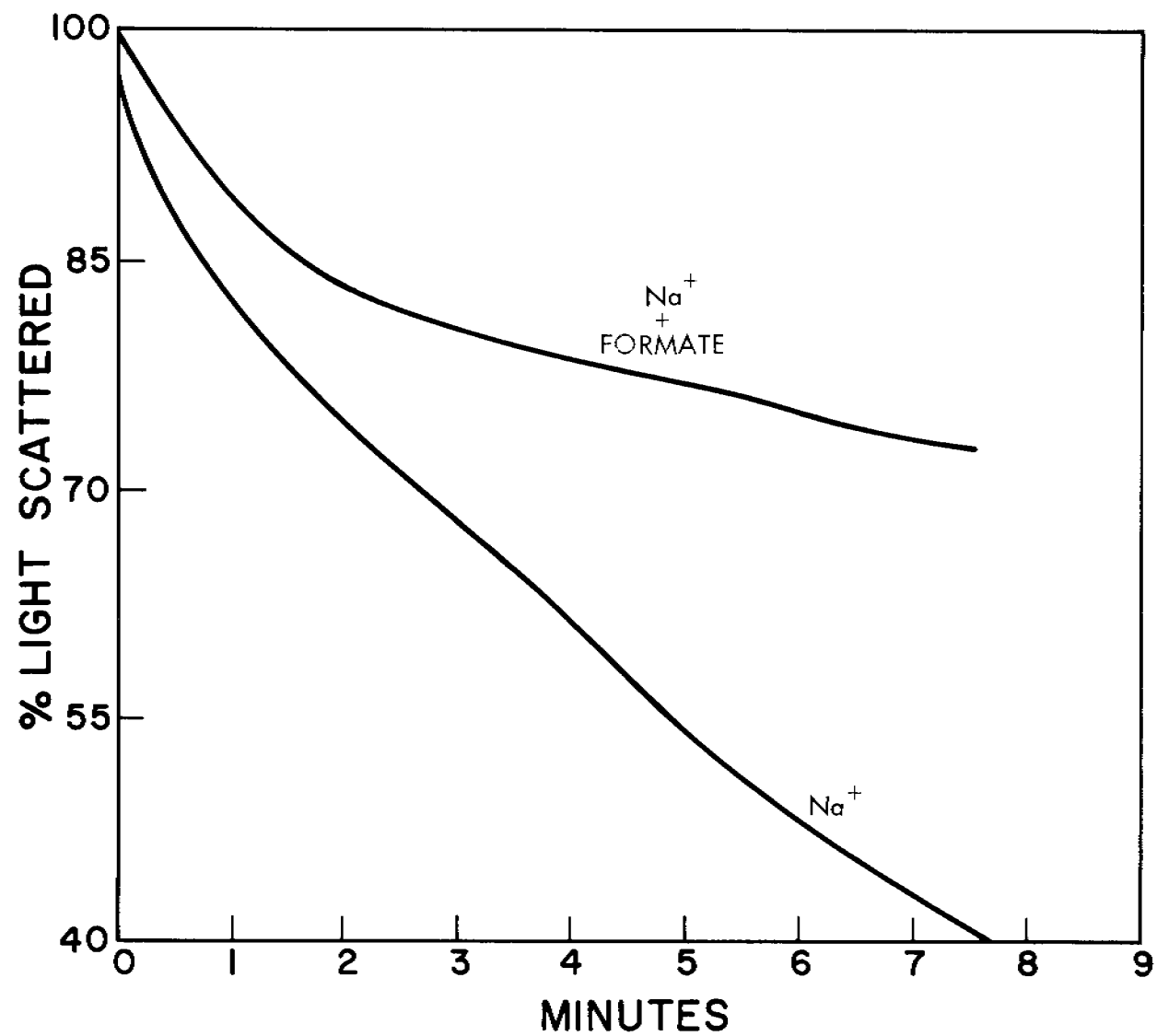


FIG. II: Effect of Formate on Na^+ Transport

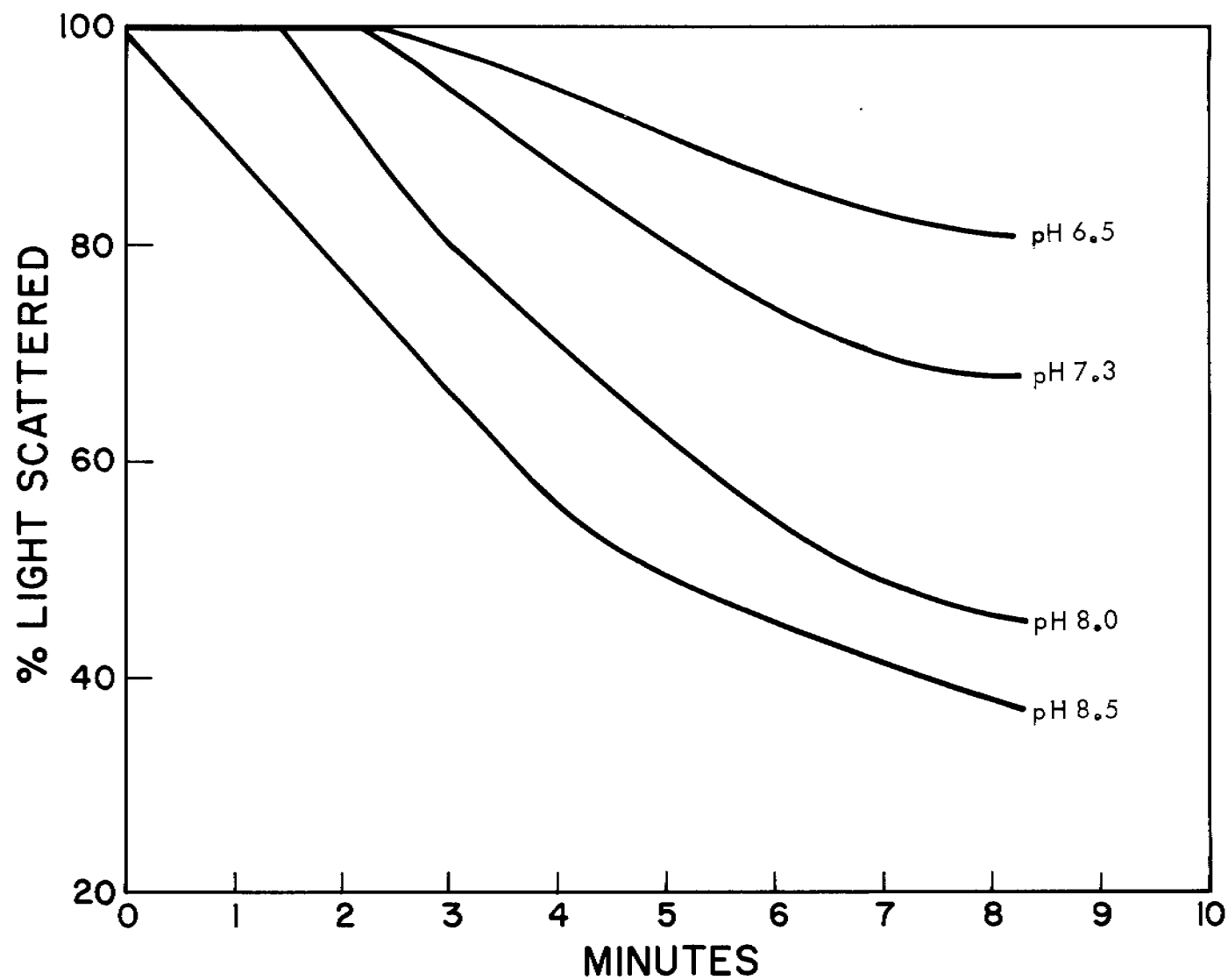


FIG. 12: Influence of pH on Na^+ Transport

TABLE 4

Influence of pH on Magnitude of Plasmolytic Response to Na^+

pH	% Increase in Light Scattering
6.5	115
7.3	125
8.0	130
8.5	115

In addition to the mechanism proposed by Conway and Moore to explain the influence of pH, it is also well known that H^+ concentration affects the kinetics of enzymes involved in metabolic energy-yielding reactions and probably the carriers concerned with transport themselves because of their enzyme-like properties. It is possible that there is a direct or indirect relationship between the rate of transport and the pH optima of those carrier or enzyme systems associated with transport, such as glycolysis.

Salt transport under anaerobic conditions

It was of interest to examine the rate of salt transport under conditions where oxygen was excluded from the cell suspension. Under anaerobic conditions only the energy derived from glycolytic reactions will be capable of transporting salts as respiration obviously cannot function in the absence of oxygen. It was hoped that this approach to the problem would further define the role of glycolysis.

Air was removed from the cell suspension by vigorously sparging with argon for 1 hour. After delivering the suspension to the cuvette, anaerobiosis was maintained by sealing the cuvette with parafilm. The oxygen electrode was immersed intermittently into the suspension contained in the cuvette to insure that anaerobic conditions were being maintained. In Figure 13 it can be seen that Na^+ transport occurred at a slow rate under anaerobic conditions (slope 1). The stock suspension was then aerated by sparging with air for 30 minutes and the rate of deplasmolysis determined under the usual aerobic conditions. Slope 2 shows that light scattering decreased by a factor of 7 over that which occurred under anaerobic conditions. The stock suspension was again deaerated by sparging with argon. Slope 3 shows that the influence of aeration was reversible as the rate of deplasmolysis was approximately the same as slope 1.

In view of the increased rate of salt transport obtained when respiration was suppressed with inhibitors, the low rate of transport observed under anaerobic conditions appears at first to be contradictory. One would expect the Pasteur effect to function in both cases and therefore result in accelerated deplasmolysis.

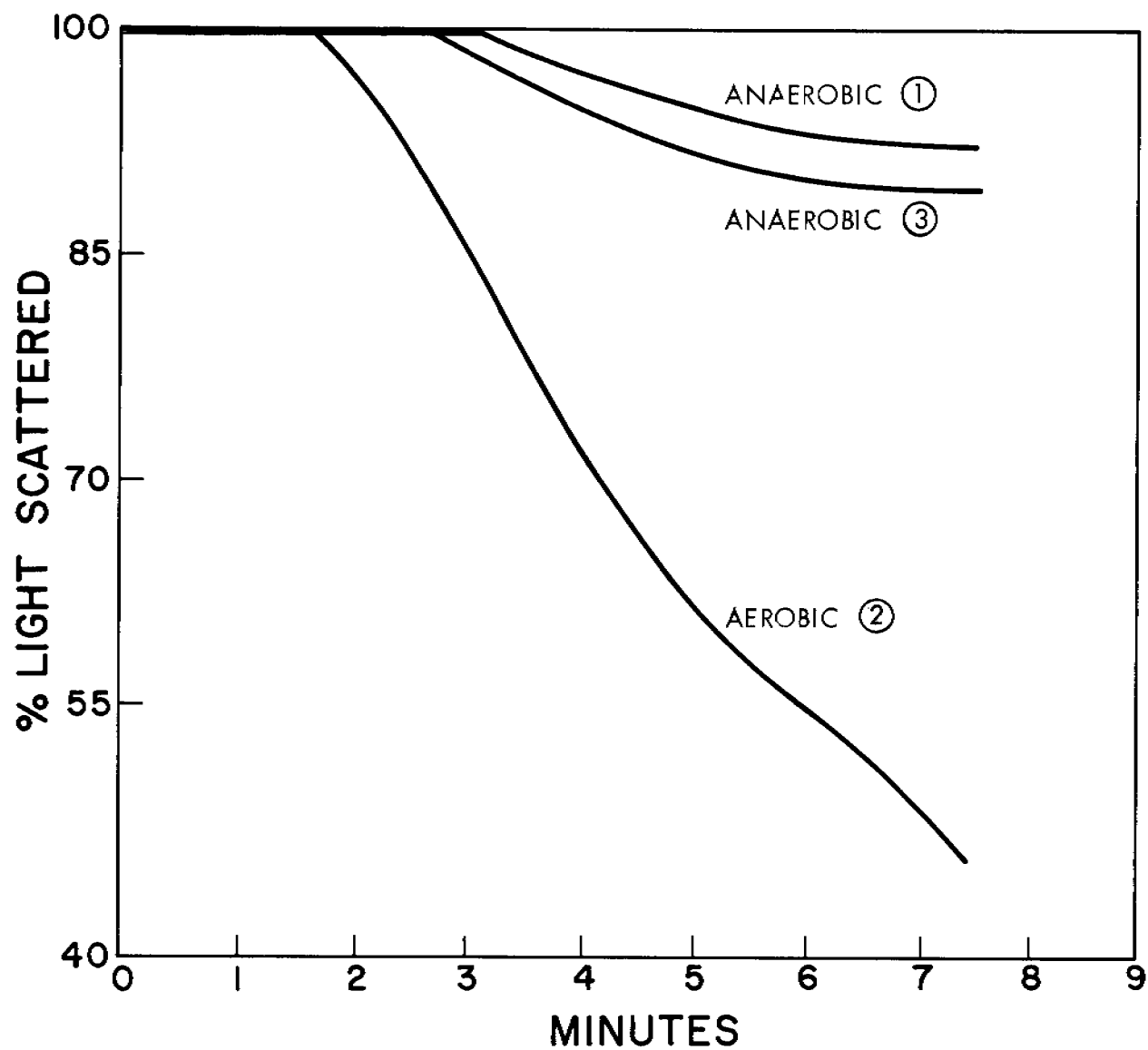


FIG. 13: Effect of Anaerobiosis on Na^+ Transport

An essential difference between conditions in the two types of experiments is that under anaerobic conditions the redox potential of the suspending medium was low, while in the inhibitor experiments, oxygen was present and hence the redox potential was correspondently high. Conway and Kernan (1955) found that the rate of Na^+ transport in yeast is directly related to the redox potential of the medium and reported that lower redox potentials slowed the rate of transport. They assigned an important role to oxidation-reduction reactions on the two sides of the cell membrane in regulating the transport of inorganic ions. The differences observed in the rates of deplasmolysis in aerobic and anaerobic environments in our experiments could be explained on this basis. Studies performed with the oxidation-reduction potentials of suspensions poised with redox dyes should be useful in examining this mechanism further, particularly in the case of respiration-inhibited cells.

Effect of aeration procedure on salt transport

In the course of examining anaerobic transport it was observed that the manner in which the cell suspension was aerated significantly influenced the rate of salt transport. The rate of deplasmolysis of a cell suspension aerated by sparging with a gas dispersion stone is shown in Figure 14. If the cell suspension in the cuvette was shaken vigorously prior to the addition of NaCl by sealing the cuvette with parafilm, rapidly inverting three times, removing the parafilm for a few seconds to permit interchange of the air in the head space and repeating the procedure five times, the rate of deplasmolysis was greatly accelerated. The same procedure, repeated without removing the parafilm intermittently, did not result in accelerated transport. Agitation alone was evidently not responsible for the increased deplasmolysis.

Further studies, using the oxygen electrode, showed that the shaking procedure increased the concentration of dissolved oxygen in the suspension and also stimulated the rate of endogenous respiration. It is difficult to explain why the respiratory rate was enhanced by increasing the content of oxygen since respiration of bacterial cells is normally linear over a wide range of oxygen concentration and does not become non-linear until oxygen is nearly exhausted. It is possible that the shaking procedure supersaturated the suspension with oxygen and respiratory stimulation might occur in this case. Whether there exists a relationship between the rate of respiration and the rate of salt transport is not known. In view of the Pasteur effect which appears to function in these cells, one would have predicted respiratory enhancement to impair the rate of transport. A critical experiment would be to determine the influence of aeration on cells in the presence of respiratory inhibitors. If the rate of transport increased under these conditions, the role of respiration could be eliminated.

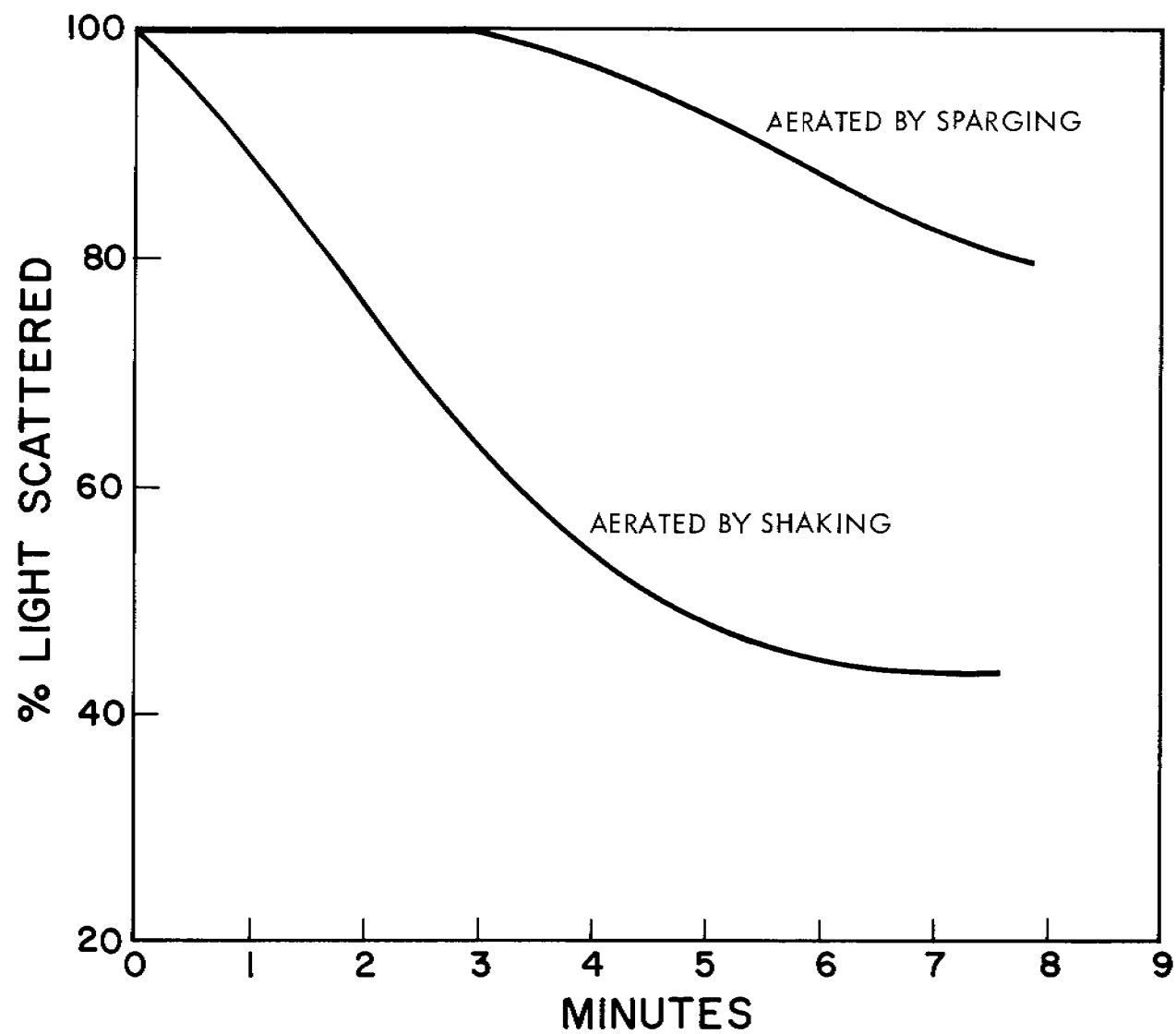


FIG. 14: Effect of Shaking Cells with Air on Na^+ Transport

Comparative salt transport between *S. marcescens* and *S. marinorubra*

The two organisms were grown in nutrient broth lacking NaCl under similar conditions, harvested at approximately the same phase of growth (early exponential phase) and resting cell suspensions prepared under similar conditions. Figure 15 shows that cells of *S. marinorubra* were markedly less permeable to salts than *S. marcescens*. Over a 7.5 minute period, cells of *S. marinorubra* did not transport either Na^+ or Li^+ ; K^+ was permeable, but the rate of transport was significantly less than with *S. marcescens*.

An attempt was also made to compare salt transport between cells of the two bacteria grown in the standard medium containing 3% NaCl. It was found, however, that the addition of salt solutions to these cells resulted in only negligible increases in light scattering. This was true even when the final concentration of salts was increased to 0.32 M. A probable explanation of these results is that the intracellular salt concentration of these cells was higher than normal. When cells are grown in hypertonic media, the internal salt content of the cell generally reaches that of its external environment. It is difficult to plasmolyze such cells because, in order to produce plasmolysis, the osmotic concentration of the external medium must exceed the intracellular osmotic concentration. To overcome this difficulty, it will be necessary to repeatedly wash the cells to leach salts from the cytoplasm of the cells. Ideally, the washing medium should be distilled water to obtain maximum leaching, but cells grown in media of high osmolarity have a tendency to lyse in distilled water, due to the concentration gradient. Hence, an impermeable substance such as sucrose may have to be added to the washing solution to make this procedure feasible.

The results obtained in the medium lacking NaCl were encouraging in that a significant difference in permeability is evident between the two bacteria. As discussed earlier, this suggests a true genetic difference in membrane structure and/or function but further studies will be necessary to elucidate the nature of this observed difference.

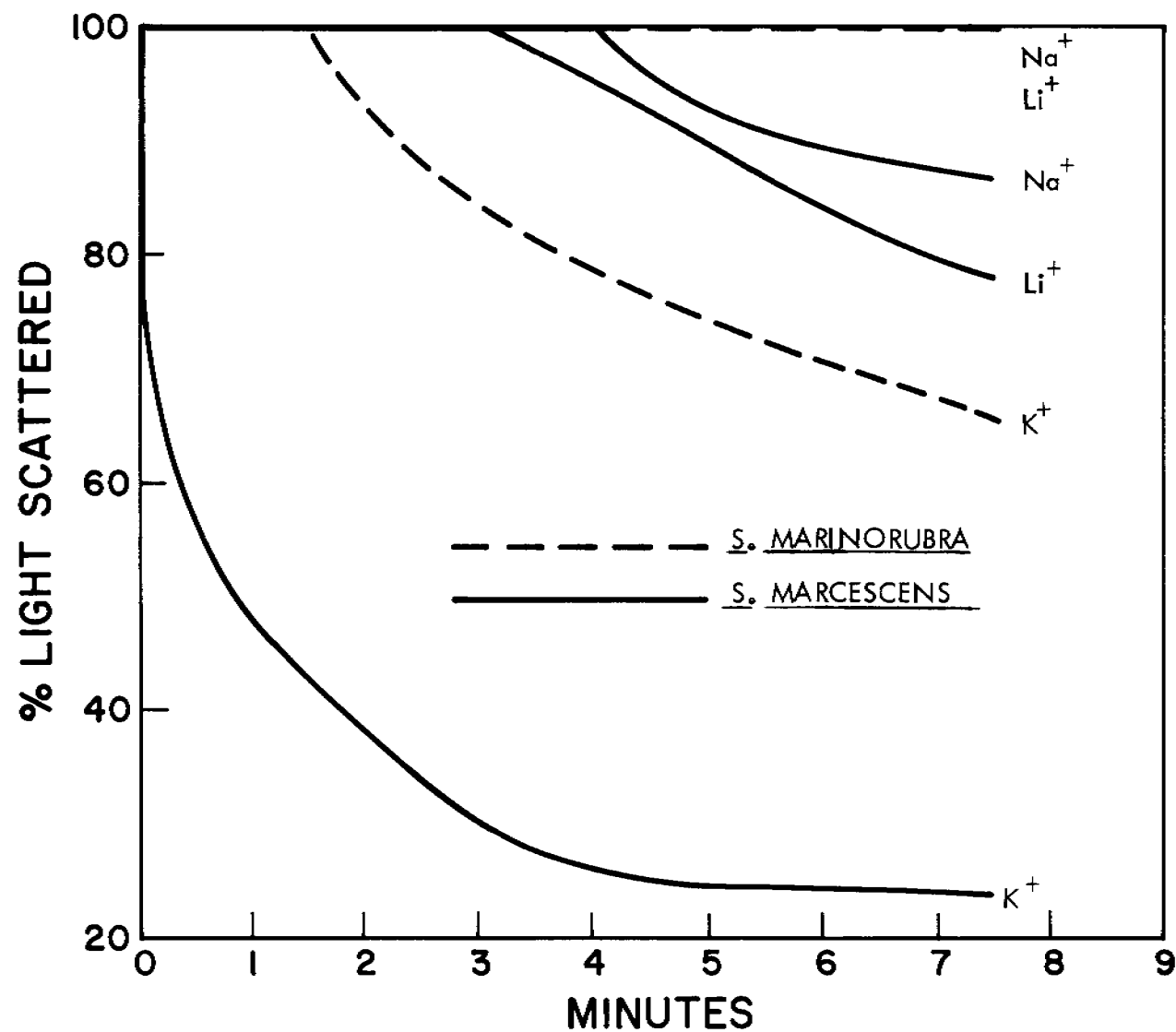


FIG. 15: Comparative Salt Transport Between *S. marnorubra* and *S. marcescens*

CONCLUSIONS

1. Based on a large variety of randomly selected biochemical tests, the metabolism of S. marcescens is identical to that of S. marnorubra. S. marnorubra is considerably more tolerant to NaCl and requires 1-2% NaCl for optimum growth.
2. Resting cells of S. marcescens and S. marnorubra plasmolyze and scatter more light in the presence of the chloride salts of Na^+ , K^+ , Li^+ , Mg^{++} , Ca^{++} and Sr^{++} . Cells of both organisms are relatively impermeable to the bivalent cations for 15-30 minutes but rapidly deplasmolyze with the monovalent cations. S. marnorubra is markedly more impermeable to the monovalent cations than S. marcescens.
3. The magnitude of the plasmolytic response and the rate of deplasmolysis are influenced by the phase of growth at which the cells were growing when harvested. The earlier in the growth cycle, the more resistant to deplasmolysis and the greater the plasmolytic response.
4. Metabolic inhibitor studies suggest that deplasmolysis is energy-dependent and that the glycolytic dissimilation of the endogenous reserve material, glycogen, provides the energy for salt transport. The inhibition of glycolysis retarded salt transport while respiratory inhibitors accelerated salt transport, suggesting that inhibition of respiration produces a Pasteur effect.

Further evidence of the Pasteur effect is indicated from studies which showed that an increase in the respiratory rate of cells provided with formate resulted in a decreased rate of salt transport.

5. The rate of salt transport is influenced by pH - the lower the pH the slower the rate of deplasmolysis.
6. Cells transported salts more slowly under anaerobic conditions than in the presence of air.
7. Vigorous shaking of the cell suspension in the cuvette increased the concentration of dissolved oxygen, stimulated respiration and resulted in accelerated salt transport.

RECOMMENDATIONS

Based on the results obtained during this study, the following recommendations are made for future investigation:

1. It will be important to measure the intracellular salt content of *S. marcescens* and *S. marinorubra* to determine whether the impermeability to salts exhibited by *S. marinorubra* is truly due to some aspect of membrane function or largely reflects a higher intracellular salt content. A higher internal osmotic pressure might explain the lower rates of salt transport with monovalent cations. Flame photometry would be useful for this purpose. This technique should also be used to correlate rates of deplasmolysis with actual net salt uptake values.
2. Studies should be performed to examine the salt transport characteristics of both organisms grown in media containing NaCl, particularly at salt concentrations that support equivalent amounts of growth. The internal salt content and composition of such cells should be correlated with rates of deplasmolysis to clarify interpretation of results.
3. An independent biochemical means of measuring the proposed Pasteur effect should be carried out to confirm data obtained with inhibited cells and cells respiring formate.
4. Salt transport should be examined in cells that are depleted of glycogen reserves and compared to those containing large amounts of glycogen to elucidate the role of endogenous metabolism in actively transporting salts. The further use of metabolic inhibitors and possibly labeled isotopes should be used to elucidate the enzymatic pathways involved in transport and to examine the coupling of glycolysis to salt transport.
5. The role of oxygen in accelerating salt transport should be examined in greater detail. The quantitative measurement of oxygen concentrations obtained during aeration procedures should be used to determine whether supersaturation occurs. The influence of aeration on the redox potential of cell suspensions should be determined and correlated with changes in oxygen concentration.

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